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On the role of rRNA tertiary structure in recognition of ribosomal protein L11 and thiostrepton

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ABSTRACT

Ribosomal protein L11 and an antibiotic, thiostrepton, bind to the same highly conserved region of large subunit ribosomal RNA and stabilize a set of NH_4^+-dependent tertiary interactions within the domain. *In vitro* selection from partially randomized pools of RNA sequences has been used to ask what aspects of RNA structure are recognized by the ligands. L11-selected RNAs showed little sequence variation over the entire 70 nucleotide randomized region, while thiostrepton required a slightly smaller 58 nucleotide domain. All the selected mutations preserved or stabilized the known secondary and tertiary structure of the RNA. L11-selected RNAs from a pool mutagenized only around a junction structure yielded a very different consensus sequence, in which the RNA tertiary structure was substantially destabilized and L11 binding was no longer dependent on NH_4^+. We propose that L11 can bind the RNA in two different 'modes', depending on the presence or absence of the NH_4^+-dependent tertiary structure, while thiostrepton can only recognize the RNA tertiary structure. The different RNA recognition mechanisms for the two ligands may be relevant to their different effects on protein synthesis.

INTRODUCTION

A protein may contact several different helix or loop regions of an RNA. To the extent that the protein depends on a precise configuration of such features, the tertiary structure of the RNA can become a determinant of protein recognition. Only for tRNA recognition by cognate synthetases has this potential connection between RNA tertiary structure and protein recognition been explored. For example, yeast phenylalanine-tRNA and aspartyl-tRNA synthetases both rely on the tRNA tertiary structure to hold recognition features in the correct positions (1,2), though the latter synthetase is less demanding and also seems to rely on tRNA tertiary interactions to stabilize a D stem recognition feature (3).

A number of ribosomal proteins may similarly require specific rRNA tertiary structures. *Escherichia coli* S4 (4), L4 (5), L11 (6) and yeast L25 (7) all recognize rRNA domains containing junctions of three to five helices and in some of these cases the junction conformation is constrained by unusual base pairs that are needed for protein binding (7,8). We have been interested in the role of tertiary structure in L11 binding to the conserved region of the large subunit ribosomal RNA shown in Figure 1; the same domain is also bound by an antibiotic, thiostrepton. Evidence for a set of unique 'tertiary' interactions contained within the 1051–1108 sequence and specifically stabilized by NH_4^+ and Mg^{2+} (in preference to other ions) has come from thermodynamic studies of this RNA fragment (9). Ligand binding and RNA melting experiments also show that L11 and thiostrepton specifically stabilize the NH_4^+-dependent tertiary structure (10–12). Thus L11 and thiostrepton must both recognize the RNA tertiary structure at some level, though mutations disrupting the tertiary structure have a much larger effect on thiostrepton binding affinity than on L11 affinity (13).

To look further at how strictly L11 and thiostrepton require a specific tertiary folding, we report in this paper a series of *in vitro* selection experiments. Our approach has been to make pools of RNA either with a uniform level of mutations through the 1051–1108 sequence or with high levels of mutations at bases surrounding the junction (referred to as degenerate and patch randomization respectively, in a recent review of *in vitro* selection methods (14). The two selections produced RNAs with very different tertiary structure stabilities and ligand binding properties. On the basis of these experiments, we suggest that the L11 protein binds the RNA in two different modes, both of which may be functionally relevant.

MATERIALS AND METHODS

DNA templates for transcribing pools of mutated RNAs were prepared by first chemically synthesizing a 99mer corresponding to nucleotides 1029–1127 of *E.coli* ribosomal RNA (15); portions of the sequence were randomized to different degrees by using appropriate mixtures of phosphoramidites. This DNA was amplified using the following two primers:

5′ ACGTCGACTAATACGACTCACTATAGGACGATGTGGGAAGG
5′ GATGGATCCGCGCAGGCCGA

The underlined sequences are *Sal*I and *Bam*HI restriction sites, respectively, and the upper sequence contains a promoter for T7 RNA polymerase. The 3′ 14 or 15 nucleotides of the two sequences are identical or complementary to the rRNA sequence, respectively. 90 pm of 99mer template were used in PCR reactions (30 cycles, 30 × 100 µl). The product DNA was gel purified and then transcribed with T7 RNA polymerase to obtain the starting material for selection. The sequence complexity of the initial

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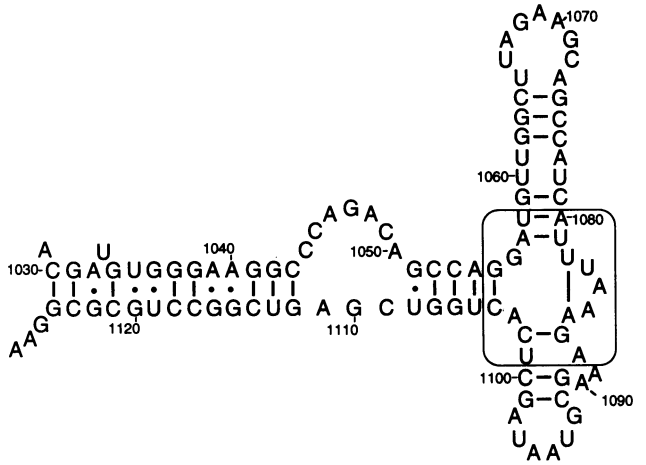

Figure 1. Secondary structure of the 23S rRNA domain binding L11. The sequence and numbering is that of the *E.coli* rRNA (15); base pairing is indicated only where conserved. The box encloses 17 nucleotides randomized in some of the RNA pools.

pools was thus on the order of 10^{13}. Sequencing of unselected RNAs indicated that the chemical synthesis had some bias towards incorporation of pyrimidines (~30% each among mutants) over purines (~20% each).

Filter selections were carried out with renatured *E.coli* L11 protein (0.28 μM) or thiostrepton (Calbiochem, 25 μM) and renatured RNA (2- to 10-fold times protein concentration or 0.5- to 3-fold the antibiotic concentration) approximately as described before for filter binding assays (6). The buffer was 30 mM Tris pH 7.6, 175 mM KCl, 20 mM $MgSO_4$; 7 mM 2-mercaptoethanol was included with L11 and 5% DMSO with thiostrepton. Incubation of the complexes was at 0°C for 10 min in 50 μl volumes. Filters (Schleicher & Scheull, 0.45 μ) were soaked in the same buffer before filtration of the complex. Filters were not rinsed after applying the complex, since both L11 and thiostrepton complexes with RNA are quickly removed from the filter by such treatment. The filter was air dried for 2 min, cut into small pieces and extracted with 400 μl phenol saturated with 30 mM Tris pH 7.6, 1 mM Na_2EDTA. The extracted RNA was transcribed with reverse transcriptase (15 min at 42°C with 20 U AMV reverse transcriptase from Life Sciences, in 50 μl with 20 pm primer annealed with the RNA at 55°C for 10 min and 50 mM Tris pH 7.6, 40 mM KCl, 6 mM $MgSO_4$ and 4 mM dithiothreitol) and then amplified with 30 cycles of PCR using *Taq* DNA polymerase. The DNA product was gel purified and transcribed for a subsequent round of filter selection. Polymerase chain reaction amplification before cloning used *Pfu* DNA polymerase (Stratagene) to reduce the error rate. DNA was cut with *Bam*HI and *Sal*I and ligated into pUC18 cut with the same enzymes. Single colonies of HB101 cells were selected on ampicillin-containing plates and grown in 1.5 ml of culture and plasmid was isolated by a modified boiling method (16). An Applied Biosystems model 373A automated DNA sequencer was used to obtain sequences by the fluorescent terminator method, using reagents and protocols from Applied Biosystems.

Nitrocellulose filter binding assays were carried out as previously described (6), at 0°C unless otherwise specified. The binding buffer was 30 mM Tris–HCl pH 7.6, 175 mM KCl, 20 mM $MgSO_4$ and 7 mM 2-mercaptoethanol. Titrations shown in Figure 4 were also carried out in buffers with NH_4Cl or NaCl substituted for KCl.

RNA transcripts were purified by gel electrophoresis and melting profiles taken in a temperature-controlled spectrophotometer as previously described (9).

RESULTS

Selections from RNA pools mutagenized over the entire binding domain

The RNA fragment shown in Figure 1, nucleotides 1029–1127 of the *E.coli* 23S rRNA, binds to L11 protein with affinity on the order of 10^7 M^{-1}, as measured by a filter binding assay (6). Nuclease protection and fragment binding studies have defined the sequence 1051–1108 as the principal protein recognition site (6,12,17,18), though the fragment 1041–1114 binds L11 several-fold more tightly than does 1051–1108 (L. Laing & D.E.D., unpublished observations). The same RNA region is also specifically bound by an antibiotic, thiostrepton, in the filter binding assay; the affinity is in the order of 10^6 M^{-1} and is the same for the 1051–1108 RNA fragment as for larger fragments (11,13,19). To carry out *in vitro* selection experiments on this region, we chemically synthesized two DNA 99mers corresponding to the sequence 1029–1127. The reagents used for nucleotides 1043–1112 contained either 85 or 92% of the correct base and an equimolar mixture of the other three bases. The unmutagenized segments of the DNA were used to hybridize primers for PCR amplification, as described in Materials and Methods.

The objective in setting up the pool of mutagenized RNAs was to generate all possible compensatory changes preserving the RNA tertiary structure (and thus the protein or antibiotic binding sites) within the protein binding domain. The possibility of a three base interaction within this RNA has been suggested (18) and we have found that up to four concerted base changes may be needed to maintain the tertiary structure stability (20), so we considered that the pool should contain at least all possible five base mutations. The number of different sequences of length N carrying n mutations is simply

$$M(n) = \frac{N!}{(N-n)!n!}3^n \qquad 1$$

The first term is the combinatorial formula for the number of ways of selecting n objects from a larger set of N distinguishable objects; 3^n gives the number of possible variants among any one set of bases altered at n specific positions, assuming that all three base substitutions are equally likely. This function is plotted in Figure 2A. A minimum of ~10^{10} molecules is needed to obtain all possible combinations of mutations at 1– 5 sites (a pool of randomly generated sequences would have to have several times this number to insure that >90% of the sequences are present at least once.)

The level of mutagenesis used to create the pool of RNAs is critical. If the level of mutagenesis is too low, there will be a higher probability of selecting wild type sequences than any RNAs with interesting mutations; conversely, a mutagenesis level that is too high will result in few molecules that retain ligand recognition. The fraction of molecules with n mutations, given a probability r of finding the wild type base at any one position, is

$$F(n,r) = \frac{N!}{(N-n)!n!}r^{(N-n)}(1-r)^n \qquad 2$$

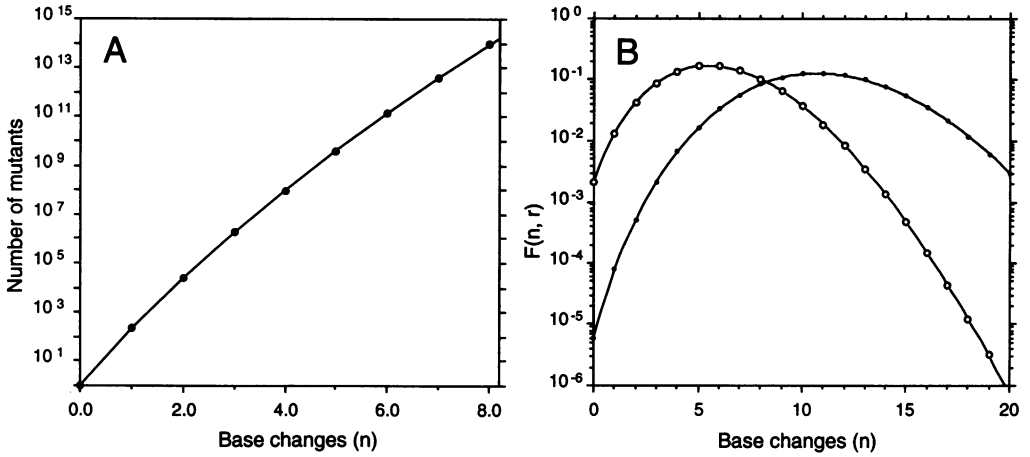

Figure 2. Design of RNA pools for *in vitro* selection. (**A**) Equation **1** of the text is plotted for an RNA length N = 70. (**B**) Equation **2** of the text is plotted for N = 70 and r = 0.85 (●) or 0.92 (○).

This is plotted in Figure 2B for r = 0.85 and 0.92, the two mutagenesis levels chosen for these selections. A pool of 10^{13} molecules is large enough to contain nearly all possible sequences with as many as five mutations at both mutagenesis rates. More than half of the molecules in the more highly mutagenized pool should contain 12 or more mutations.

After five rounds of selection using the pool with r = 0.85, 9/10 RNAs selected with L11 were the wild type sequence and 6/8 sequences selected with thiostrepton were wild type. Of the five mutation sites found in these selected RNAs, four are unlikely to weaken L11 or thiostrepton binding (at positions 1044C, 1090A, 1096A and 1111A) (13) and the fifth (1099C→U) disrupts a conserved base pair. Five unselected RNAs were sequenced as well and gave the expected level of variation, 10–15 mutations per RNA. The fraction of wild type sequences is surprisingly high and suggests that only a small fraction of mutants retain ligand binding ability.

Selections with the less highly mutated RNA pool gave more interesting results. After five rounds of selection with either L11 or thiostrepton, about half of the sequences were wild type (6/12 L11-selected sequences and 5/9 thiostrepton-selected) and the remainder had one or two mutations. Several of the selected mutations either disrupt conserved base pairs or make changes known to weaken ligand binding (13). Selection was therefore continued for another five rounds to ensure that virtually all of the selected sequences would have wild type or better binding affinities. The results were quite striking (Table 1). For the L11-selected sequences, 18/20 had 1051G→A. Tests of this RNA in the filter binding assay show that it increases the L11 binding affinity by a small but significant factor (1.8 ± 0.2-fold, averaged from four titrations) but does not alter the thiostrepton binding constant within experimental error (data not shown). Mutations at only three additional sites were found. Of 20 thiostrepton-selected sequences, two were wild type and the rest had 1–4 mutations; the most frequent number of mutations was two (eight sequences). The mutations were distributed among a total of 16 sites, half of which were outside of the 1051–1108 sequence sufficient for thiostrepton recognition. Selection of all but one of the variants within the 1051–1108 region (1107G→C) is consistent with phylogenetic variation of the sequence and with known binding properties of site-directed mutations (13), as summarized in Table 1. Of particular interest is the selection of several mutations at 1061U and at 1089A–1090A. Phylogenetic variants at these two positions increase the stability of the tertiary structure by as much as 25°C (20). The two mutations at 1107G were unexpected, since they disrupt a base pair within a helix. These were tested in the filter binding assay and bound >7-fold less tightly than the wild type sequence. Five other thiostrepton-selected RNAs were also tested and had affinities within 40% of wild type (data not shown). The significance of these results for thiostrepton recognition of RNA tertiary structure is pursued further in the Discussion.

Selection from RNA pools mutagenized in the junction region

Measurements of L11 and thiostrepton binding affinities with a series of site-directed mutants have shown that mutations affecting RNA recognition by both ligands are clustered around the junction region of the structure (13). We therefore targeted this region for mutagenesis by preparing two more pools of RNA, with either completely random base incorporation or a 70% bias towards wild type within 17 nucleotides of the junction and surrounding base pairs (see Fig. 1). Selections with the completely random set were abandoned after six rounds, since no specific binding could be detected with either L11 or thiostrepton. A total of 17 rounds of selection with L11 or 27 rounds with thiostrepton were carried out with the pool mutagenized to 30%. Only the L11-selected RNAs showed binding affinity comparable to wild type RNA when pools were tested in the filter binding assay.

RNAs were sequenced starting after eight rounds of L11 selection. 5/21 RNAs sequenced from the 8th cycle were wild type. The remaining RNAs (1–7 mutations each) showed a fairly random distribution of mutations among 17 sites and bound L11 weakly when tested individually. After 11 rounds of selection, no wild type sequences were found among 20 sequences. Some of these bound L11 weakly when tested individually ($K < 4\ \mu M^{-1}$); the remainder had affinities within a factor of two of wild type ($7–22\ \mu M^{-1}$) and are listed in Table 2. Though the distribution of mutations is less random than seen with the RNAs sequenced after eight rounds, there is still no consensus sequence or pattern of compensatory mutations.

Table 1. RNAs selected from pool with 8% mutagenesis at 1043–1112[a]

	Comments
L11-selected sequences	
A1044C (2)	5′ to binding domain
A1051G (18)	Common in eubacteria
C1073A	Disrupts potential base pair
	U substitution has no effect on K_{L11} (13)
G1095A	Universally conserved
	U substitution has no effect on K_{L11} (13)
Thiostrepton-selected sequences	
U1043C (2), A1044C (2), U1045C, G1047U, A1048G	Sequences 5′ to binding domain
G1109C, U1110G (4), A1110G, U1112G, U1110GA	Sequences 3′ to binding domain
A1051G (8)	Common in eubacteria
A1061U, C1061U	Disrupts potential base pair
	A1061 found in some thermophilic archaebacteria
	A or G1061 stabilizes tertiary structure (20)
U1089A, U1090A	Variable portion of internal loop
	Some substitutions stabilize 3°C structure (20)
G1096A	Universally conserved
	U substitution does not affect K_{L11} or K_{TS} (13)
A1097U, C1097U	C substitution common
	A substitution does not affect K_{L11} or K_{TS} (13)
C1107G, U1107G (2)	Disrupts 1052/1107 base pair
	Thiostrepton binding is weakened

[a]All mutations found in 20 L11-selected and 20 thiostrepton-selected RNAs are listed. The letter preceding the nucleotide number is the sequence found and the following letter is the wild type sequence. Numbers in parentheses give the number of times the mutant was found. Two L11-selected sequences were wild type and 12 others were identical (A1051 only). Two thiostrepton-selected sequences were identical (U1110G, U1107G).

A clear consensus was apparent in sequences taken from rounds 13–17 (Table 2). The same 1051G→A mutation picked up from the first selections (Table 1) was present in all sequences and all sequences also carried a deletion or single base change in or near the 7 nucleotide hairpin loop (1066–1072). Two of the RNAs also had two bases deleted from the 6 nucleotide hairpin loop (1093–1098), leaving the sequence GUAA, a member of the GNRA class of stable tetraloops (21). By the last round of selections, all the sequences also carried a 1044C→A mutation in the internal loop outside of the 1051–1108 domain. The A1051G and A1044C mutations and loop deletions and are all outside of the 17 mutagenized nucleotides and were probably introduced by polymerase errors during PCR amplification.

Starting in round 11, unusual sequences were found that could not be aligned with the wild type sequence; they were presumably generated by errors during PCR amplification and/or T7 polymerase transcription. These RNAs have very stable structures (in melting experiments), bind to L11 and compete to some degree with 1029–1127 RNA in binding L11 (data not shown), but there is no strong similarity to the wild type structure or consensus among the sequences or predicted secondary structures. The relevance of these sequences to the L11 binding mechanism will have to await further experimental work.

Stability of the L11-selected RNA

The three consensus substitutions of pyrimidines for purines within the junction region are individually known to disrupt the RNA tertiary structure. Both phylogenetic evidence and previous site-directed mutagenesis have shown that L11 requires Watson–Crick complementary bases at positions 1082/1086 (8); the 1086A→U mutation, in the absence of a compensatory change at 1082U, disrupts this pairing and destabilizes the RNA tertiary structure in melting experiments (9). 1088A is universally conserved and 1088A→U also disrupts the RNA tertiary structure (20). Finally, position 1056 is always a purine; its mutation to U destabilizes the RNA tertiary structure (20). It is not obvious how the loop deletions or A1044C and A1051G mutations could compensate for the junction mutations to maintain the RNA tertiary structure. We have therefore examined one of the selected RNAs in melting experiments to see if there is any evidence that the tertiary structure has been preserved.

The unfolding of the RNA tertiary interactions can be detected by comparison of the melting profiles in buffers containing Na^+ or NH_4^+ as the monovalent cation (10,20). This is shown for the 1029–1127 RNA fragment in Figure 3A. The tertiary transition is clearly seen in the NH_4^+-containing buffer as the extra

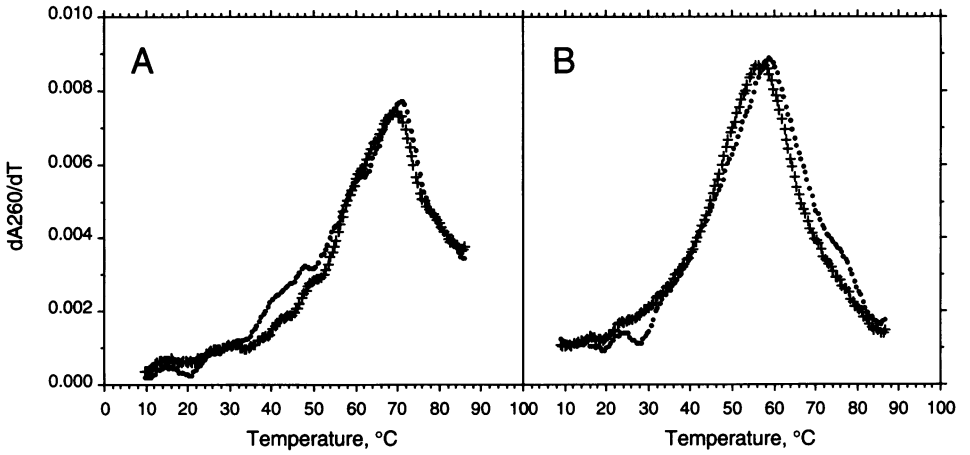

Figure 3. Melting behavior of 1029–1127 RNAs. (**A**) wild type sequence or (**B**) sequence 2 of round 17 selection (Table 2). Melting profiles were taken in buffer containing 3 mM $MgCl_2$, 10 mM MOPS pH 7.0 and 100 mM of either NH_4Cl (●) or NaCl (+).

Table 2. L11-selected RNAs from pool mutagenized within the junction[a]

Selection Round	1050	1060	1070	1080	1090	1100
	CCAGACAGCCAGGAUGUUGGCUUAGAAGCAGCCAUCAUUUAAAGAAAGCGUAAUAGCUCACUGGU					
11						
1		CUG		UC		C
2	A	U		U		
3	U			C CU C	C U	
4	A	UG		U U	U	
5		C		U C		
6	-----	C C			CA	
7				-- C	U	
8	---	AU		C CC	A	
9				C C	C	AA
10		UA	C	C CCCG	U	
11	A					
12	A	U		U		
13						
1	A	U	U	U		
2	AU	A C	---	U C		
3		A	---	U U	U	
16						
1	A	A C	---	U C	--	
2		A U	A	UC	C	
3		A C	C	U C		
17						
1	AU	A C	---	U		
2	A	A C	---	U U	--	
3	A	A U	---	U C		
4	A	A C	---	U U	A	
		C		C		
Consensus	A	A U	---	U U		
	CCAGACAGCCAGGAUGUUGGCUUAGAAGCAGCCAUCAUUUAAAGAAAGCGUAAUAGCUCACUGGU					

[a]The wild type (*E.coli*) rRNA fragment sequence is shown at the top and bottom. Partially randomized regions are underlined. Sequence changes in RNAs selected in different rounds are shown; dashes indicate deleted bases. Sequence 11 of round 11 was isolated twice. The 'consensus' sequence summarizes the most frequent base changes in rounds 13–17.

hyperchromicity below ~50°C. The difference between the two curves is not as dramatic as it is in the case of the smaller 1051–1108 RNA fragment, probably because the extended extra helix and internal loop unfold to some degree in the same temperature range. Tertiary structure unfolding in the 1051–1108 RNA fragment also has a characteristically low hyperchromicity at 280 nm (20) and this is also observed in the 1029–1127 RNA fragment (data not shown).

The same melting experiment with sequence 2 from round 17 (Table 2) is shown in Figure 3B. There are several indications of a drastic overall destabilization of the RNA structure. At low temperatures the change in absorbance with temperature approaches zero for the wild type RNA, but is substantial for the selected sequence. There is no longer any indication of a separate, NH_4^+-stabilized tertiary structure. (The small difference between the two curves at high temperature is unexplained, but it cannot be related to the low temperature tertiary unfolding in wild type RNA. Shift of the tertiary unfolding to higher temperatures simultaneously stabilizes the entire secondary structure (12,20). The peak of the whole curve has been shifted to lower temperatures by 12–14°C and comparison of melting curves taken at 260 and 280 nm show the disappearance of a transition with low A280/A260 at low temperatures (not shown). None of the mutations should have a very large effect on the stability of the secondary structure: shortening of either hairpin loop should decrease the corresponding stem melting temperature by only ≤1°C and the G1051A substitution should not change the melting temperature of this stem by >1°C (22). We therefore attribute the destabilization to extensive disruption of 'tertiary' interactions. Our previous analysis of the 1051–1108 RNA melting pathway suggested that elimination of the set of tertiary interactions and disruption of 1082U/1086A base pair stacking with the other helices within the junction could destabilize the structure to the degree seen here (9).

Ligand-binding properties of selected RNAs

To see if L11 binds the Table 2 consensus sequence by a different mechanism than wild type RNA, we measured binding constants in buffers containing Na^+, K^+ or NH_4^+. Previous work has shown that the L11–RNA binding affinity increases with substitution of monovalent ions in the order $Na^+ < K^+ < NH_4^+$, as the ions stabilize the RNA tertiary structure; the ratio of affinities in Na^+ and NH_4^+ at 25°C is ~7 (10). Titrations carried out with the same selected RNA used in the Figure 3 melting experiments under these same conditions are shown in Figure 4. There is no detectable difference in L11 binding affinity between experiments in the different ions.

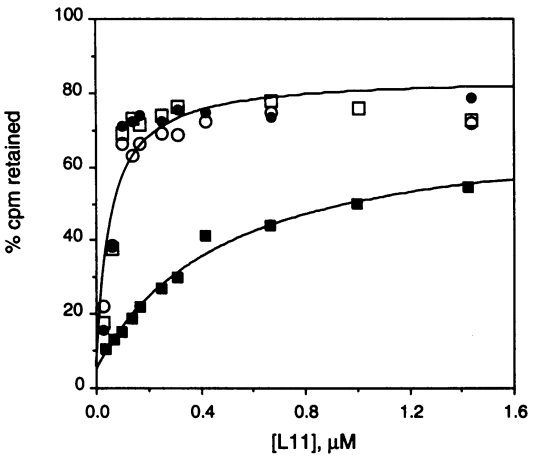

Figure 4. Binding of L11 to selected RNA (round 17, sequence 2 in Table 2). Filter binding assays were carried out as described in Materials and Methods, in buffers containing NH_4^+ (●), K^+ (○) or Na^+ (□). The curve is a least squares best fit of a binding isotherm to the data taken in Na^+. The binding constant is 19 μM^{-1}, with a background of 6.0% and a maximum retention of 85%. Shown for comparison is a titration of the wild type RNA sequence in buffer containing Na^+ (■), with K = 2.1 μM^{-1} (taken from ref. 10).

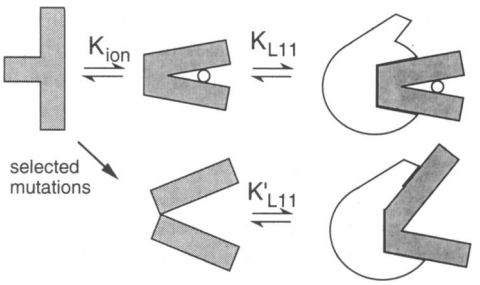

Figure 5. Hypothesized interactions of L11 and NH_4^+ with RNA. Specific binding of NH_4^+ (small circle) to the RNA stabilizes a structure recognized by L11 (top row). Mutations in the RNA destabilize the tertiary structure and allow binding with a different set of contacts (bottom row). See text for further discussion.

Relative to wild type RNA, the selected RNA binds nearly an order of magnitude more tightly in the presence of Na^+. We conclude that L11 is binding this RNA by a different mechanism that does not require the monovalent ion-dependent tertiary structure. There was no detectable binding of this same selected RNA to thiostrepton (not shown).

DISCUSSION

Selections from pools uniformly mutagenized through the binding domain

In vitro selections with RNA sequences carrying random mutations are a potentially powerful way to generate the equivalent of a phylogenetic data base, that is a set of homologous sequences that all retain a single functional property. A problem with this approach is that repeated rounds of selection may amplify very small differences in binding affinity. For instance, two RNAs differing by only 1.5-fold in ligand affinity may differ >50-fold in their enrichment after 10 rounds of selection. Thus a binding free energy difference that is nearly undetectable by direct measurement can have a large influence on the distribution of sequences in the final pool. The narrow 'window' of binding constants in the final pools of selected RNAs has to be taken into account when interpreting our selection data. Despite this limitation, the selections from RNA pools mutagenized throughout the 1043–1112 sequence suggest that both thiostrepton and L11 require a specific structure encompassing all or most of this domain.

The thiostrepton-selected sequences have substantially fewer mutations (most probable number is 2) than the pool average (most probable number, 5) and the distribution of 'hits' within the mutagenized region is non-random. Only within the 12 nucleotides 5′ and 3′ to the known 1051–1108 binding domain were the mutations randomly distributed; there were 15 hits at eight of these 12 nucleotides. This region must be largely irrelevant to thiostrepton binding, consistent with the fact that the 1051–1108 RNA fragment alone binds thiostrepton normally (11,19). The remaining 17 hits are concentrated in a few selected regions of the molecule and all of these can be rationalized as mutations that either stabilize or do not affect the RNA structure. The most frequently found thiostrepton-selected mutation is A1051G, which was also selected by L11. This mutation does not have any detectable effect on the stability of the RNA tertiary structure (20) or thiostrepton binding constant; we presume that there is some enhancement too small to be picked up within the error of the filter binding experiment (±30%). Disruption of the potential 1061U–1077A base pair by G1061 or A1061 mutations dramatically increases the RNA tertiary structure stability, as does alteration of two phylogenetically variable bases in an internal loop, 1089AA (20). Thus the selected mutations at 1061, 1089 and 1090 may enhance thiostrepton binding by stabilizing the RNA tertiary structure. Mutations in the hairpin loop bases 1096A and 1097U do not affect thiostrepton binding significantly (13), though 1095A is critical for thiostrepton recognition (13,23). We have recently determined the structure of this hairpin loop by NMR methods (S. Huang and D.E.D., unpublished) and find it highly structured with some hydrogen bonding similarities to GNRA tetraloops (21). 1097U is completely unstacked and excluded from the structure; therefore substitution of C or A at this position may have little effect on the loop conformation. 1096A is tightly stacked with 1095A but not hydrogen bonded; therefore the G1096A substitution will probably not perturb the loop structure. Lastly, the mutations at 1107G are presumably irrelevant to thiostrepton recognition, since the RNAs carrying these mutations bind much more weakly than wild type.

The L11 selections from the same pool of sequences also suggest that L11 recognizes a specific RNA structure. The selected RNAs contain only three other mutations in combination with the A1051G mutation that enhances L11 binding slightly. In contrast to the results with thiostrepton, there is evidently no region of the RNA that is completely neutral with respect to L11 recognition (with the caveat that <2-fold differences in binding free energy are detected in this selection). Footprint experiments have not suggested any direct L11 interactions with the large internal loop (1044–1050/1109–1112) that is irrelevant to thiostrepton recognition. However, selection against mutations in this loop is consistent with our finding that sequences 1029–1127 and 1041–1114 do bind L11 significantly better than 1051–1108 RNA (L. Laing and D.E.D., unpublished observations).

The conclusion suggested by these selection results is that both L11 and thiostrepton are sensitive, at some level, to base changes throughout the 1051–1108 RNA domain. Even two or three base compensatory changes that preserve or slightly enhance the ligand binding constants must be exceedingly rare or non-existent. The conclusion is consistent with a previous site-directed mutagenesis study (13), in which only four out of 42 single and double mutations preserving the conserved secondary structure had thiostrepton relative binding affinities >80% of wild type; mutations at three of those sites were picked up here (1061U, 1089AA and 1097U). Seven mutations from the same set had L11 affinities >80% of wild type and two of those (1073A and 1095A) were found among the selected RNAs. The conclusion is also consistent with melting studies showing that L11 and thiostrepton both stabilizes a tertiary structure encompassing the entire 1051–1108 domain (11,12).

The only other attempt to delineate a protein binding site by *in vitro* selection from a partially randomized pool of sequences was made with the Rev protein (24). In that case, a very high rate of mutagenesis (65% wild type at any one position) was applied to a 66 nucleotide domain and only two short segments, forming an internal loop, showed a much reduced level of mutagenesis. That we selected sequences with much reduced levels of mutations compared to the initial pool, even with a low rate of mutagenesis, also indicates that L11 and thiostrepton require a much more extensive RNA structure than does Rev protein.

Are there two modes of L11–RNA interaction?

It appears that the second set of selections attempted with L11 inadvertently turned into an '*in vitro* evolution' experiment, in which mutations not present in the starting pool accumulated in successive rounds through errors in amplification of the selected sequences. The consensus sequence obtained after many rounds of selection contains the A1051G and A1044C mutations previously found, but also includes mutations violating the expectation that L11 binding is optimized by stable tertiary interactions. Melting experiments show that the tertiary structure has been eliminated and L11 binding has lost its characteristic dependence on monovalent ion type that indicates stabilization of the tertiary structure. Compelling evidence that L11 recognizes a NH_4^+-dependent tertiary structure in the wild type RNA has already been cited (10,12). We are thus faced with the paradox that L11 can recognize the tertiary structure of the wild type RNA but is indifferent to its absence in the selected sequence variants. L11 is an unusually specific RNA binding protein, as we cannot detect interactions of it with tRNA or homopolymers (6,12). It is therefore difficult to discount the selected sequence as irrelevant to the functional RNA binding mechanism of L11.

To resolve this apparent contradiction we propose that L11 has more than one binding mode. A cartoon presenting this hypothesis is shown in Figure 5. In the wild-type RNA, NH_4^+ stabilizes a specific structure in the RNA (K_{ion}) and this structure holds the recognition features in an optimum configuration for L11 recognition (K_{L11}). The substantially destabilized RNA structure in the consensus RNA sequence suggests that this RNA may adopt alternative conformations more easily than the wild type. For instance, disruption of the 1082U/1086A pair may create a more flexible junction and U1056G, U1088A and the loop deletions may disrupt tertiary hydrogen bonding or specific ion binding sites. We imagine that the remaining RNA secondary structure adopts a conformation that is able to make additional (or alternative) protein contacts not available to the more rigid wild type structure; the increased free energy of interaction compensates for the larger entropy of the RNA to produce a complex with about the same overall binding free energy as the wild type sequence (K'_{L11}).

This hypothesis of two binding modes is supported by the previous observation that all of the mutations known to destabilize the RNA tertiary structure reduce L11 binding by an unexpectedly small factor. We have studied nine single base changes that destabilize the 1051–1108 RNA tertiary structure by at least 3.5 kcal/mol at 0°C (corresponding to a shift in the tertiary structure T_m to ≤0°C) (20). Only one of these mutants (1085A→U) binds L11 even 10-fold more weakly at 0°C; the other eight bind with 2- to 5-fold reduced affinities (ΔG weakened by 0.4–0.9 kcal/mol). This disparity in the free energy changes argues that the protein is able to find an alternative way (or ways) to contact the RNA when the tertiary structure is weakened. How different the protein contacts in the presence and absence of tertiary structure might be is a subject for further investigation. The two binding modes are unlikely to be completely different sets of RNA contacts, since A1051G and A1044C were selected in both modes. Elsewhere we will describe N-terminal deletions of the L11 protein that weaken binding to the selected consensus sequence but do not affect NH_4^+-dependent binding to RNAs with stable tertiary structures (Y. Xing and D.E.D., unpublished observations). These protein fragments may help delineate the possible RNA binding modes.

All mutations that weaken the RNA tertiary structure also weaken thiostrepton binding by >10-fold. (Very weak thiostrepton binding constants cannot be measured in the filter binding assay, so a more precise measure is not possible.) Thiostrepton binding to the selected consensus sequence is also undetectable. We conclude that the alternative binding mode is unavailable to thiostrepton. This difference in binding mechanism is potentially related to the different activities of the two ligands, that is L11 stimulates protein synthesis while thiostrepton inhibits it. As proposed by Cundliffe (25), L11 may promote needed RNA conformational changes during the ribosome cycle while thiostrepton may slow the ribosome by trapping a single RNA conformation.

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